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Estuarine, Coastal and Shelf Science xxx (2016) 1–10

Contents lists available at ScienceDirect

Estuarine, Coastal and Shelf Science

journal homepage: www.elsevier.com/locate/ecss

Review of the use of *Ceramium tenuicorne* growth inhibition test for testing toxicity of substances, effluents, products sediment and soil

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ARTICLE INFO

Article history:
Received 5 January 2016
Received in revised form
16 August 2016
Accepted 4 October 2016
Available online xxx

Keywords:
Ceramium tenuicorne
Antifouling paints
Pulp mill effluents
Toxicity
Soil
Sediment

ABSTRACT

A growth inhibition test has been developed based on two clones of the red macroalga *Ceramium tenuicorne*, one originating from 7 PSU and the other from 20 PSU. The species can be adapted to different salinities and the test can be carried out between 4 and 32 PSU. This test became an ISO standard in 2010 (ISO 107 10) for testing of chemicals and water effluents. In this study new and published data has been compiled on toxicity of single substances, waste waters from pulp mills, leachates from antifouling paints, harbour sediments and soil used for maintenance of leisure boats. The results show that the alga is sensitive to both metals and organic compounds and to biocides used in antifouling paints. By testing leachates from antifouling paints these could be ranked according to their toxicity. Similarly, the toxicity of waste waters from pulp mills was determined and the efficiency of secondary treatment evaluated. Further, the test method proved useful to test the toxicity in sediment samples. Sediments from small town harbours and ship lanes were shown to be harmful and compounds originating from antifouling paints were responsible for a large part of the inhibiting effect. The alga proved to be sensitive to contaminants leaking from boat yard soil. The growth inhibition test is a robust test that has high repeatability and reproducibility and easily can be applied on water, soil and sediment samples without being too costly. The species is found worl-wide in temperate waters, which makes the results relevant for large areas. In the Baltic Sea *C. tenuicorne* is the most common red alga species and is thus particularly relevant for this area. The overall results show that contaminants from boat activities and the use of antifouling paints in particular pose a threat to the environment.

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1. Introduction

More and more chemicals are produced, placed on the market and used in society. Information on the hazards they pose to human health, biota and the environment has been insufficient and for this reason regulations have been introduced to control unneccessay use of harmful substances and products. The REACH regulation (EC 2006/2006) has been in force since 2007 in EU and aims to improve environmental protection through better and earlier identification of the intrinsic properties of common chemical substances. Biocides used in products are regulated in the Biocidal Product Regulation (EU BPR) (EC 528/2012) since September 1, 2013 in EU. In both REACH and BPR the risk to humans and the environment is estimated by a number of tests. Concerning environmental effects a test battery of test organisms representing three trophic levels, e.g. a microalga, an invertebrate and a fish is usually used. The most commonly used tests are the standardized tests on growth inhibition to microalga, acute test with Daphnia and an acute test with fish. It is optional to use other tests as long as it can be shown that these tests are reliable and reproducible, e.g. one of the standardized tests by ISO or OECD. Other important criteria for being a suitable test are that the test organism is relevant for the investigated area, sensitive, fast and can be performed at a low cost (e.g. Chapman, 1995, 2000; Nendza, 2002). If the same test organism and test can be used in different compartments like water, sediment and soil it would be an advantage for comparison of toxicity. The use of Ceramium growth inhibition test in different compartments is reviewed in this paper with focus on evaluating harmful effects from boat activities.

Leisure boats are particular common in the Nordic countries where about half (Eklund et al., 2013) of the estimated European 6 million (ICOMIA, 2007) boats are found. Biocides are added to antifouling paints to prevent attachment of fouling organisms to the hull. Too much fouling will reduce the speed and may disrupt the manoeuvrability of the boat and become a safety hazard.

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http://dx.doi.org/10.1016/j.ecss.2016.10.009
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However, the active agents in these paints are not only harmful to fouling organisms but may also affect non-target organisms and negatively affect the coastal ecosystem (e.g. Andersson and Kautsky, 1996; Alzieu, 1991, 2000; Kautsky and Svensson, 2003; Antizar-Ladislao, 2008; Thomas and Brooks, 2010). Tributyltin (TBT) is the most well-known biocide that has been used as active ingredient in antifouling paints (e.g. Almeida et al., 2007). Due to its efficacy TBT-paint was widely used in the 70 and the 80-ies but when its endocrine effects were discovered in the 80 ies (Alzieu et al., 1986, 1989) it soon became prohibited within EU (Council Directive 89/677/EEC). Diuron replaced TBT as active agent in antifouling paints (Almeida et al., 2007; Antizar-Ladislao, 2008). However, this substance turned out to have carcinogenic effects and was prohibited in the 90-ies in Sweden (Kemi, 1993). Irgarol a commonly used booster biocide that affects the photosynthetic system (e.g. Mohr et al., 2008) and zinc pyrethione affecting crustaceans were prohibited in Sweden in 2001 (Kemi, 1998). Today copper is the most commonly used biocide in antifouling paints.

Even if biocides used in antifouling products largely have been restricted (e.g. TBT since 1989 (Council directive 89/677)), residues are still found in water, sediment and in soil all over the world (e.g. Oliviera et al., 1999; Hoch, 2001; Thomas et al., 2001; Fent, 2006; Konstantinou and Albanis, 2004; Turner, 2010). The biocides from antifouling paints will eventually end up in the sediment and pose a risk to this ecosystem. Spread of biocides from antifouling paints will also occur at boatyards. In the northern countries leisure boats are used only a few summer months and during winter stored on land. Before launching next season old antifouling paint is removed and new is applied. Scrapings end up on the soil and concentrations of contaminants well above guidance values are found (Eklund and Eklund, 2014). The potential toxicity in water, sediment and soil of from biocides originating from antifouling paints and other types of waste waters needs to be evaluated.

2. Material and methods

2.1. The Ceramium growth inhibion test

2.1.1. The test organism Ceramium tenuicorne

Ceramium tenuicorne Kützing Waern is a red macroalga belonging to a genus found in all oceans and particularly abundant in temperate waters (Lüning, 1990). It is growing in both marine and brackish waters and is able to survive far up in the Bothnian Bay at a salinity of only 1 PSU (Bergström et al., 2003). It can be found from the surface down to very low light conditions, 2–3 μmol s^{-1} m^{-2}, on stones and as epiphytes on larger algae. The growth inhibition test is based on two clones, one from the marine environment and one from brackish water in the Baltic Sea. The marine clone was isolated by Dr Jan Rueness (1978) at the University of Oslo and originates from the Oslo fjord (20–25 PSU) and has been maintained as a laboratory culture for over 40 years (Rueness, 1978). The brackish water clone was isolated by Dr Britta Eklund (2005) at the University of Stockholm about 100 km south of Stockholm (7 PSU). Complete interfertility between these two, earlier regarded as two species from the Baltic Sea and the Oslofjord (former *C. strictum* Harvey *sensu* Kylin), has been shown by Rueness and Kornfeldt (1992). DNA data has confirmed that the two entities belong to the same species, with *C. tenuicorne* as the valid name (Gabrielsen et al., 2003).

2.1.2. The test procedure

The Ceramium growth inhibition test (Bruno and Eklund, 2003; Eklund, 2005) became an ISO standard in 2010 (ISO 107 10, 2010). The principle of the test is to determine growth inhibition as reduction in growth rate, measured as length at different concentrations of chemical/effluent water compared to a control during a seven day exposure period. The two available clones means that the test can be performed in a wide salinity range and after adaption may be used in salinities between 4 and 32 PSU (Eklund, 2005; Ytreberg et al., 2011; Heijerick et al., 2012; Macken et al., 2012).

All tests in the studies reported in this paper were performed according to the ISO standard 107 10. Briefly, the sample and control waters in the test series were enriched with nitrogen (N) (3.46 mg/L), phosphorus (P) (0.78 mg/L) and iron (Fe) (0.10 mg/L). Top pieces (2–3 mm in length) of the alga were exposed to different dilutions for 1 week. The length of the algae was measured at the start and at the end of the test. All tests were performed in sterile polystyrene Petri dishes, in four replicates for each test concentration. During exposure, the dishes were kept at 22 °C ± 2 °C, a light regime of 10 h darkness and 14 h light at a light intensity of 70 ± 7 μmol m^{-2} s^{-1}. One test consists of five to seven test concentrations plus a control with four replicates in each. The algal growth rates in the different test solutions were estimated and compared with that of a control and EC50 values were calculated by linear interpolation by using the software REGTox.xls V6.4 (http://eric.vindimian.9online.fr).

Adaptation and preparation of test waters, sediment and soil samples are described below.

2.2. Single substances

Twelve single substances have been tested with the Ceramium growth inhibition test. Six of these, i.e. tributyltin (TBT), diuron, irgarol, zinc-pyrithione, copper and zinc, are compounds that have or is being used in antifouling paints (Karlsson and Eklund, 2004; Eklund, 2005; Karlsson et al., 2006; Ytreberg et al., 2010). The remaining tested substances were the metals cadmium (Cd), chromium (Cr), mercury (Hg), and the organic compounds phenol, 3,5-dichlophenol and 2,4,6-tribromophenol. The substances were diluted in five to seven different concentrations in enriched sea water at 7 or 20 PSU and were tested according to the ISO standard 107 10. The organic biocides used in antifouling paints and Hg and Cd were tested in five test concentrations and EC50 with the corresponding 95% confidence interval from the four replicates was calculated. The remaining six substances were tested several times and a mean of the EC50 values with standard deviation was calculated.

2.3. Waste waters

Waste waters from pulp mills using different types of secondary treatment, e.g. aerated logoon, sedimentation, bioreactor, have been tested with the Ceramium growth inhibition test. The waste waters were collected between 1999 and 2005 and the tests were performed in salinities that corresponded to the recipient of the respective pulp mill, i.e. between 4 and 20 PSU. Each waste water was diluted in five to seven test concentrations with enriched sea water of the appropriate salinity and each test concentration was tested in four replicates. The EC50 with the corresponding 95% confidence intervals was calculated.

2.4. Antifouling paints

2.4.1. Paints tested

Altogether, leachates from 15 antifouling paints have been tested (Karlsson and Eklund, 2004; Karlsson et al., 2006, 2010). Four paints were based on copper. Two of these were intended for use on ships (larger than 12 m) Antifoulung Olympic, (Hempel) and Interspeed, (International) and the other two intended for use on

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leisure boats at the Swedish West coast, Fabi (International) and Cruiser Superior (International). The latter antifouling paint contained copper and irgarol 1051 and was used as reference paint in all studies. To one of the leisure boat paints, EcoMar 2000 (Thulica), capsaicin from the pepper fruit had been added as active ingredient. The remaining nine paints all claimed not to contain biocides but to be working by physical means. Four of these paints were self-polishing, i.e. Mille light (Hempel), Micron Eco (International), Lefant SPF (Lotréc) and Cruiser Eco (International). Two paints, SafeBoatSkin (Sailway) and Aurora VS721 (Aurora Marine Industries Inc.) were so-called polymer waxes and one had a waxy scale structure SSC-44 (US Gloss). One paint, Lefant H2000 (Lotréc AB), claimed to be physical growth repellant, one paint is based on silicone according to the Intersleek 700 system (www.intersleek700.com) and one Vc17m Eco (International) was based on teflone.

2.4.2. Preparation of leachate waters from antifouling paints

Five cm^2 of each paint was applied according to the information on the respective can on the back side of a petri dish. When the paints had dried each piece was placed in 500 ml 7 PSU autoclaved sea water. After 1 h the leachate was disregarded to remove possible remnants of solvents. Another 500 ml was added and the painted pieces were left to leach for 14 days in room temperature on a shaking table to simulate water movement and in darkness to avoid growth of photosynthetic organisms. This water was used for testing growth inhibition to the red macroalga *C. tenuicorne*. All test waters were prepared in triplicates except for two paints where the test was performed on only one leachate sample.

2.5. Sediment

2.5.1. Sediment samples

Superficial (0–2 cm) sediment samples were collected from small town harbours and natural harbours used by leisure boats in the Stockhlm archipelago (Eklund et al., 2008; Eklund et al., 2010) and on the West coast of Sweden (Eklund et al., 2016) In addition sediment samples were collected in the ship lane at different distances (1, 15, 25 50 and 65 km) from Stockholm city. All samples were placed in cold (4 °C) and in darkness for a maximum of 2 weeks before testing.

The investigated places in the Stockholm area were two reference harbours (RH), a natural harbour frequently visited by leisure boats (NH), a large marina with 1500 boats (LM), a small marina with 250 boats (SM) and Stockholm city (SC). The salinity in the Stockholm archipelago is low and the tests with *C. tenuicorne* were conducted with the brackish water clone at 5 PSU.

At the West coast of Sweden top sediment (0–2 cm) were sampled from 12 small boat harbours and 4 natural harbors. The natural harbours are places which form a natural sheltered anchoring place far from the civilization. In this study the marine clone was used and the test was conducted in 20 PSU.

Sediment samples along the ship lane into Stockholm were sampled in 2010. Five regions were selected at different distances (1–65 km) from Stockholm center. From each region three samples were collected for chemical analyses and toxicity testing. Metal analyses were performed on each replicate whereas analysis of organotin compounds (tributyltin (TBT), dibutyltin (DBT) and monobutyltin (MBT)), the sum of the16 most common polyaromatic hydrocarbons (Σ16 EPA PAH), the carcenogenic fraction of the PAHs and toxicity testing was performed on pooled samples from the three replicates. All chemical analysis were performed by the acredited laboratory ALS with standardized methods.

2.5.2. Preparation of leachates for testing of sediment

Sediment concentration series were prepared according to the following: 16 g of native sediment (on wet weight basis) was diluted in sterile natural sea water of appropriate salinity (5 PSU or 20 PSU) up to 100 ml in an Erlenmeyer flask to reach a sediment concentration of 160 g/L (1:1). Subsequent sediment concentrations (1:2, 1:4, 1:8 and 1:16) were prepared by transferring 50 mL of a higher concentration to a new Erlenmeyer flask followed by the addition of dilution media up to 100 mL. The Erlenmeyer flasks with different dilutions were placed on a shaking table for 24 h. The sediments were allowed to settle for at least 12 h. The overlaying water was then filtered through 0.45 μm and these elutriates were, after addition of nutrients (final concentration: 3.5 mg N/L, 0.9 mg P/L, 103 μg Fe/L), used as test samples.

2.6. Soil

2.6.1. Soil samples

Soil from a boatyard used for winter storage of 200 leisure boats since 1955, was sampled for toxicity testing (Eklund et al., 2014). Four station levels, A, B, C and D located 70, 90, 120 and 160 m from the shore were chosen for the sampling. At level A, B and C, three replicates of soil sample, approximately 10 m apart from each other were collected. At station D only one replicate was sampled. At each location both surface (0–0.5 cm) and subsurface (19–21 cm) samples were collected.

2.6.2. Preparation of leachate water for testing of soil

All samples were filtered through a 3 mm mesh to remove larger stones before leaching. In the leaching experiment, soil (300 g) and MilliQ water (500 ml) were added to pre-cleaned glass beakers. The subsamples from sites A to C were pooled, i.e. 100 g soil from each of the three replicates was added to the respective beaker. This procedure was conducted for both surface and subsurface soil samples. After 24 h of incubation with constant shaking the soil particles were left to precipitate for an additional 72 h. The water phase was filtered (0.45 μm) and stored refrigerated (4 °C) before used in ecotoxicological tests. The stock leachate water was adjusted to 7‰ with NaCl and enriched with nitrogen (3.46 mg/L), phosphorus (0.78 mg/L) and iron (0.10 mg/L). Dilution series were made of the leachate water by dilution with filtered (30 mm) autoclaved enriched (N 3.46 mg/L, P 0.78 mg/L and Fe 0.10 mg/L) natural seawater of 7 PSU. The growth inhibition test with *C. tenuicorne* was performed according to the ISO standard 10710 (2010).

The soil samples were also tested with the Microtox test (ISO, 2007) where effect on inhibition of bio-luminescence on *Vibrio fisheri* is measured and the larvae development test with *N. spinipes* (Breitholtz and Bengtsson, 2001). The test procedures of these organisms are presented in Eklund et al. (2014).

3. Results

3.1. Toxicity of single substances to growth of Ceramium tenuicorne

According to the ISO test with *C. tenuicorne* TBTO was found to be most toxic of the tested substances and only 0.49 μg/L was needed to inhibit growth by 50%. Irgarol was approximately half as toxic as TBT with an EC50 of 0.96 μg/L. Diuron, zinc pyrethione, 2,4,6 tribromophenol and copper had similar toxicity with EC50 between 2 and 3.4 μg/L. The least toxic substance of all 12 tested was phenol with EC50 of 38 000 and 46 000 μg/l in 7 and 20 PSU respectively (Table 1). Of the compounds used in antifouling paints Zinc, which is not considered as a biocide but often used as a binder of the paint, was the least toxic substance used in antifouling paints

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Table 1
Effects of single substances on growth of the red macroalga *Ceramium tenuicorne* in 7 or 20 PSU sea water. New data and data compiled from [a]Karlsson and Eklund 2004, [b] Eklund 2005, [c] Karlsson et al., 2006, [d] ISO ringtest 2006 (10 or 12 tests), [e]Ytreberg et al., 2011.

Compound	Salinity PSU	No of tests	No of test concentrations	No of replicates/test concentration	EC50 (µg/l)	Conf. int 95%
TBTO [c] (AF)	7	1	5	4	0.49	0.49–0.50
Irgarol 1051 [c] (AF)	7	1	5	4	0.96	0.51–0.98
Diuron [c] (AF)	7	1	5	4	3.4	2.9–3.8
Zinc pyrethione [a] (AF)	7	1	5	4	3.3	1.8–4.9
2,4,6-tribromophenol	7	1	5	4	2.1	1.9–2.2
2,4,6-tribroophenol	20	1	5	4	4.6	4.3–4.8
Mercury (Hg^{2+})	7	1	5	4	14	11–18
Mercury (Hg^{2+})	20	1	5	4	16	13–18
Cadmium (Cd^{2+})	7	1	7	4	122	98–161
Cadmium (Cd^{2+})	20	1	7	4	570	490–640
					EC50	**SD**
Phenol	7	5	5–7	4	38 000	6400
Phenol	20	5	5–7	4	46 000	7000
3,5-dichloophenol	7	3	5	4	2300	560
3,5-dichloophenol [d]	20	12	5	4	2300	550
Zinc (Zn^{2+}) [b, d] (B)	7	5	5	4	24	5.1
Zinc (Zn^{2+}) [b, d] (B)	20	16	5	4	47	18
Copper (Cu^{2+})[b, e] (AF)	7	5	5–7	4	3.1	1.1
Copper (Cu^{2+})[b] (AF)	20	3	5–7	4	11	2.6
Chromium (Cr^{6+})	7	3	5	4	900	200
Chromium (Cr^{6+})	20	5	5	4	4700	890

AF – biocide used in antifouling paints.
B – substance used as binder in antifouling paints.

with an EC50 of 24.4 µg/L.

3.2. Toxicity of waste waters from pulp mills

Two of the mills, Smurfit kappa and Munksund, were sampled before and after a bioreactor used as secondary treatment. After treatment the toxicity was considerably reduced in both mills and for Smurfit kappa from an EC50 of 4.7–30% and for Munksund from 19 to 47% a reduction in toxicity by 84 and 60%, respectively. The toxicity of the other waste waters was between 23 and 61 with Mönsterås letting out the least toxic waste water (Table 2).

3.3. Toxicity of antifouling paints

The most noteworthy is that many of the so called physical working paints produced leachates as toxic as the ones containing biocides (Table 3, Karlsson and Eklund, 2004; Karlsson et al., 2006, 2010). The leachate from SSC-44 gave an EC50 of 0.19% and was about as toxic as the leachate from the reference paint, Crusier superior containing copper and irgarol. The leachates from five of the so called biocide free paints, Cruiser Eco, Micron Eco, Lefant SPF, Lefant H2000 and Mille light all leaked something that affected the growth of *C. tenuicorne* and had EC50 from 0.35 up to 2.8% leachate. Only five of the paints did not produce leachates that were toxic to growth of *C. tenuicorne* at the highest tested concentration.

Of the copper based antifouling paints Cruiser superior used as reference paint produced the most toxic leachate with an EC50 of 0.17%. Fabi, the coating intendedfor use on leisure boats, was similar in toxicity as the paint Interspeed intended for use on ships, with EC50 0.28 and 0.33% leachate respectively. The leachate from the other ship paint, Antifouling Olympic, was approximately half as toxic and the EC50 was 0.62%.

3.4. Toxicity of sediments

The most toxic sediment was found in the harbour of a small marina (SM) in Stockholm with EC 50-ies between 1 and 6.4 g dw/L, in Stockholm city (SC) with EC50 of 2.9 g dw/L (Table 4B, Eklund and Karlsson, 2010) and in the three innermost regions (1–25 km) of the ship lane with EC 50 values between 2.1 and 5.2 g dw/L (Table 4B). The toxicity of the two regions furthest out in the ship lane was much less toxic with EC50 of 25 and > 29 g dw/L,

Table 2
Effects from complex effluent waters from pulp mill industries to growth of the macroalga *Ceramium tenuicorne*. Algae adapted to the salinity representative for the recipient of the respective pulp mills were used in the test.

Industry	Effluent treatment	Sampling year	Salinity in recipient and test	% Effluent	
			PSU	EC50	95% CI
Mönsterås	Aerated lagoon	1999	7	61	52–67
Smurfit kappa (bb)	Before bioreactor	2001	4	4.7	3.5–5.8
Smurfit kappa (ab)	After bioreactor	2001	4	30	26–33
Munksund (bb)	Before bioreactor	2002	4	19	16–22
Munksund (ab)	After bioreactor	2002	4	47	43–51
Mörrum	Bioreactor	2003	8	37	33–40
Karlsborg (Billerud)	Aerated lagoon	2004	4	27	21–32
Vallvik (03)	After sedimentation	2005	4	23	19–27
Vallvik (05)	After sedimentation	2005	4	28	24–32
Värö	Bioreactor	2005	20	23	21–26

(bb) = before bioreactor; (ab) = after bioreactor.

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Table 3
Growth inhibition to the red macroalga *Ceramium tenuicorne* in leachates (2 weeks of leakage) from antifouling paints in 7 PSU artificial seawater. Compiled data from Table 2 in Karlsson and Eklund (2004), Table 2 in Karlsson et al. (2006) and Table 1 in Ytreberg et al. (2010).

Paint	Function/active ingredient	Growth inhibition to ceramium
		Mean of 3 tests EC_{50} (%) (sd)
Cruiser superior[a] (International)	Copper thiocyanate (19%) Irgarol 1051 (3.3%)	0.17 (0.06)
SSC-44 (US Gloss Europe AB)	Waxy with scale structure	0.19 (0.03)
Fabi Reg. nr 3959 (International)	Cuprous(I)oxide (6%) Boats <12 m on the Swedish West coast.	0.28 (0.07)
Interspeed 617 (IS) Reg. nr 3897 (International)	Cuprous(I)oxide (56%) Intended for ships >12 m. Not Bothnian Bay.	0.33 (0.10)
Cruiser Eco (International)	Polishing	0.35 (0.27–0.46)[b]
Micron Eco (International)	Polishing	0.57 (0.03)
Antifouling Olympic 86951 (AO) (Hempel)	Cuprous(I)oxide (39%) Intended for ships >12 m. Not Bothnian Bay.	0.62 (0.05)
Lefant SPF (Lotréc AB)	Polishing	0.56 (0.46–0.69)[b]
Lefant H2000 (Lotréc AB)	Polishing	1.44 (0.69)
Mille light (Hempel)	Polishing	2.8 (1.73)
SafeBoatSkin (sailway)	Polymer wax	>100
Aurora VS721 (Aurora Marine Industries Inc.)	Polymer wax	>100
EcoMar2000 (Thulica AB)	Capsaicin (pepper extract)	>100
Intersleek® 700 system www.intersleek700.com	silicone	>100
Vc17mEco (International)	Teflon	>100

[a] Reference paint containing copper and irgarol.
[b] Only one test (EC50 with 95% confidence interval).

respectively. The toxicity in the ship lane correlated to the measured concentrations of contaminants where the innermost station had very high concentrations of contaminants and high toxicity, the outer two stations had lower content of pollutants and the stations in between had intermediate values both in toxicity and concentrations of pollutants (Table 4B).

The sediments from the small town harbours from the West coast of Sweden were a little less toxic than the Stockholm sediment with Hjuvik (EC50 6.9 g dw/L) as the worst and Kebal (EC50 48 g dw/L) as the least toxic sediment (Table 4A). One of the sediments from a natural harbour at the West coast showed some toxicity but three did not show any toxicity in the highest tested concentration. The reference sites in Stockholm showed some toxicity with EC values of 47 and 53 g dw/L. Due to very fluffy sediment in the NH in the Stockholm archipelago it was not possible to achieve higher test concentrations than 6.4 and 8.9 g dw/L, respectively.

3.5. Toxicity of soil

C. tenuicorne was the most sensitive organism and 0.25% of the leachate from site D was sufficient to result in a 50% growth inhibition to *C. tenuicorne*. Eight times higher concentration of the elutriate was needed to affect the development of the crustacean Nitocra and 64 times higher concentration was needed to reduce the bioluminescence from the bacteria *Vibrio fisheri* in the Microtox test (Table 5, Eklund et al., 2014). The results corresponded well with the high concentrations of contaminants related to substances used in antifouling paints, i.e. Cu, Zn, lead (Pb), organotin compounds (TBT, DBT, MBT) and irgarol (Eklund et al., 2014). The concentrations are in most cases higher and at some sites much higher than the Swedish guidance value for less sensitive land, i.e. land used for industrial purposes (Swedish guidance values for less sensitive land are for Cu, Pb and Zn 200, 400 and 500 mg dw/kg, respectively). The highest concentrations were found at site D with concentrations of16 300, 989, 18 600 mg/kg dw for Cu, Pb and Zn, respectively (Eklund et al., 2014).

On basis of measured Cu and Zn in the leachates the concentration of each metal at the EC50 level was calculated and compared to EC50 results when Cu and Zn were tested as single substances (3.1 ± 1.1 μg/L for Cu and 24.4 ± 5.1 μg/L for Zn) (Table 1). The concentrations that are higher than these values are considered to, in part, be the cause of the observed toxicity and are shown in bold figures. The results show that Zn in leachates from the surface soil at all sites is part of the toxicity and copper only at site C. However, in the leachates from the subsurface Cu is part of the cause at both site A and B (Table 6).

4. Discussion

The results of new and published data show that the growth inhibition test with the macroalga *C. tenuicorne* is working well for testing single substances, waste waters, leachates from paints, sediment and soil. The species is common along the coasts of both marine and brackish waters in temperate waters (Lüning, 1990), which makes the results highly relevant for these areas. It is particular relevant for use in the Baltic Sea since *C. tenuicorne* is the dominant algal species all the way up to the Bothnian Bay (Bergström et al., 2003). The ring-testing by several laboratories in five countries in the standardization procedure within ISO showed that the reproducibility was high with a coefficient of variation for eleven tests on Zn of 46% and for twelve test on 3,5-dochlorophenol

Please cite this article in press as: Eklund, B., Review of the use of *Ceramium tenuicorne* growth inhibition test for testing toxicity of substances, effluents, products sediment and soil, Estuarine, Coastal and Shelf Science (2016), http://dx.doi.org/10.1016/j.ecss.2016.10.009

Table 4A
Growth inhibition of the red macroalga *Ceramium tenuicorne* exposed to concentration series of sediment leachate from five locations in the Stockholm archipelago area, 5‰ and to sediment leachates from harbours used by leisure boats on the Swedish West coast, 20‰. (Compiled data from Table 4A,4B in Eklund and Karlsson, 2010 and Table 6 in Eklund et al., 2016).

Sediment					
Stockholm archipelago, 5 PSU			West coast of Sweden 20 PSU		
Location	EC50 g dw/L (95% conf. int.)	EC50% leachate (95% conf. int.)		EC50 g dw/L (95% conf. int.)	EC50% leachate (95% conf. int.)
Small marina (SM),			**Small marinas**		
Harbour basin, stn 1	6.4 (1.8–34)	4.5 (1.3–24)	Björlanda Kile	24 (18–34)	6.1 (4.5–8.7)
Harbour basin, stn 2	2.5 (1.5–4.3)	2.5 (1.5–4.3)	Eriksberg	39 (33–47)	10 (8.9–13)
Harbour basin, stn 3	1 (0.5–1.8)	1.5 (0.8–2.8)	Hinsholmskilen, inner	13 (11–15)	5.2 (4.3–6)
Harbour basin, stn 4	>11	>16	Hinsholmskilen, outer	19 (15–21)	7.2 (5.9–8.3)
20 m from uptake area	2.4 (0.57–5.5)	1.3 (0.3–2.9)	Hjuvik	6.9 (4.8–10)	2.3 (1.6–3.5)
30 m from uptake area	15 (5.9–48)	8.8 (3.6–29)	Hålkedalskilen	34 (24–54)	15 (10–23)
Large marina (LM)			Kebal	48 (47–55)	17 (16–19)
Inner part	>36	>16	Kungshamn	12 (10–13)	6.3 (5.1–7)
Outer part	>54	>16	Marstrand	11 (7.8–16)	4.3 (3–6.1)
Launching & uptake	36 (24–48)	13 (8.5–17)	Nödinge	20 (18–23)	6.3 (6–7)
Stockholm City (SC)			Wallhamn	37 (31–37)	16 (13–16)
Boat wash, enter	29 (18–44)	6.7 (3.5–10)	Önnered	22 (20–25)	11 (9.4–12)
Boat wash, exit	15 (4.9–35)	4.9 (1.6–12)	**Natural harbours**		
Stockholm City	2.9 (1.6–5.8)	0.9 (1.5–1.8)	Gluppö-Fläskön	26 (22–32)	15 (13–19)
Natural harbour (NH)			Gråholmen	>16	>16
Inner part	>6.4	>16	Store Bror	>16	>16
Outer part	>8.9	>16	Tjälleskär	>16	>16
Reference site (RS)					
Site 1	53 (44–80)	20 (17–31)			
Site 2	47 (37–72)	16 (12–24)			

- No confidence interval was possible to calculate.

of 25% (ISO 107 10). Good repeatability and reproducibility has also been shown by tests on single substances (Eklund, 2005) and on leachates from antifouling paints (Karlsson and Eklund, 2004; Karlsson et al., 2006). The species is well studied and responses on light, temperature and salinity are presented in Eklund (2005). The optimal temperature and light intensity were 22 °C ± 2 °C and of 70 ± 7 µmol m^{-2} s^{-1}, respectively and were chosen as standard procedure of the test. A light regime of 10 h darkness and 14 h light was chosen for the standard. The test may be carried out in a salinity range between 4 and 32 PSU (Eklund, 2005). The growth differs with the salinity but since the growth is compared to a control at the same salinity this compensated for (Eklund, 2005; Ytreberg et al., 2011; Macken et al., 2012). The test is robust, simple to carry out and easy to keep in culture. It is also cost efficient and one test with 5–7 test concentrations with four replicates per test concentration all together only takes one work day for one person. These qualifications should make the test a good choice in many test batteries.

4.1. Single substances

The six tested substances used in antifouling paints were the most toxic of the tested single substances. Of these the *C. tenuicorne* growth inhibition test ranked TBT as the most toxic and zinc as the least toxic and diuron, zinc-pyrethione and irgarol in between. This is similar order but not always the same as have been shown for other test species (e.g. Kobayashi and Okamura, 2002;Férnandez-Alba et al., 2002). This illustrates the need for using several test organism in risk assessment. The EC50 of *C. tenuicorne* for TBT was 0.49 µg/L. The adhesion of the alga *Porhyra yezoensis* was affected at 120 µg TBT/L and germination was affected at 8 µg TBT/L (Maryama et al., 1991). A test with *Dunaliella tertiolecta*, a planktonic microalga, resulted in an EC50 of 0.46 µg TBT/L in a 48 h test (Cheung et al., 2003). The molluscs have been shown to be the most sensitive group to TBT and effects are found on *Nucella lapillus* already at 1 ng TBT/L (Bryan et al., 1986). Effects from other molluscs and also crustaceans are reviewed e.g.by Beaumont and Budd (1984). Differences in the acute toxicities of TBT between amphipods were studied by Ohji et al. (2002) and they observed effects as low as 1.2 µg TBT/L. A thorough review of effects from TBT was made by Antizar-Ladislao (2008).

A number of acute toxicity (EC50) values of individual antifouling agents, e.g. diuron and irgarol, can be found on the bacteria *Vibrio fisheri*, the alga *Selenastrum capricornutum* and the crustacean *Daphnia magna* (e.g. Férnandez-Alba et al., 2002). The alga showed to be the most sensitive species with an EC50 of diuron at 45 µg/L. Irgarol that affects the photosynthetic system was consequently most toxic to the alga, *S. capricornutum* with an EC50 of 10.8 µg/L. Long-term effect of irgarol was found at 0.34 µg/L on periphyton and plankton during a 135 days exposure (Mohr et al., 2008). These effect values are comparable to the effect on *C. tenuicorne* with an EC50 after 7 days of exposure of 0.96 µg irgarol/L and 3.4 µg diuron/L. This makes *C. tenuicorne* approximately ten times more sensitive than *S. capricornutum*. Sea urchins are regarded as very sensitive to zinc pyrethione and EC50 of 5–9 µg/L was reported by Kobayashi and Okamura (2002) which is about the same as for *C. tenuicorne* with EC50 of 3.3 µg/L.

C. tenuicorne is also sensitive to other metals and organic compound as seen by the results in Table 1. Generally, algae are very sensitive to copper and EC50 values between 2 and 10 µg Cu^{2+}/L were reported for several species (Eklund and Kautsky, 2003). McPherson and Chapman (2000) compiled LC/EC50 values for toxicity of copper to marine and estuarine invertebrates and fish. They found the variation in sensitivity between and within all groups of species to be very high. The span for amphipods was 90 to 50 000 µg Cu/L, for bivalves 5.3 to 40 000 µg Cu/L, and for fish 460 to 58 300 µg Cu/L. The Ceramium is not always the most sensitive species and in a study of toxicity of molybdate using nine salt water species, only two species were less sensitive than *C. tenuicorne* (Heijerick et al., 2012). The authors had chosen organisms representing different groups and conclude that it is important to be able to perform tests with standardized tests to provide relevant results.

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Table 4B
Growth inhibition of the red macroalga *Ceramium tenuicorne* exposed to concentration series of sediment leachate taken from accumulation bottoms taken from the ship lane into Stockholm at different distance from the center of the city. Concentrations of contaminants in the sediment are also presented.

Site and distance (km) from Stockholm center	Ceramium growth inhibition, EC50 g dw/L (95% conf. interval)	Ceramium growth inhibition, EC50% leachate. (95% conf. interval)	TBT µg/kg dw	DBT µg/kg dw	MBT µg/kg dw	PAH Σ16 EPA mg/kg dw	PAH carc. mg/kg dw	Cu mg/kg dw (±sd)	Pb mg/kg dw (±sd)	Zn mg/kg dw (±sd)	Sediment dw in %
1 km	2.1 (1.6–2.8)	1.4 (1.1–1.8)	390	320	79	22.9	10.3	367 ± 32	288 ± 41	42 ± 2.9	15
15 km	5.2 (3.8–7.0)	3.0 (2.2–4.1)	87	37	29	2.4	1.1	74 ± 7	91 ± 3.0	14 ± 2.6	17
25 km	4.4 (3.3–5.4)	2.5 (1.9–3.1)	68	25	19	1.2	0.43	56 ± 2	61 ± 1.6	7.2 ± 0.4	17.6
50 km	25 (12–103)	13.2 (6.5–54)	26	13	13	0.5	0.14	45 ± 7.6	30 ± 6.4	3.4 ± 0.7	19
65 km	>29 (-)	>16 (-)	9.5	6.7	9.9	0.7	0.21	47 ± 6	46 ± 5.7	4.5 ± 0.8	18
Correlation toxicity/substance			−0.64	−0.55	−0.66	−0.53	−0.54	−0.54	−0.62	−0.64	

- No confidence interval was possible to calculate.

The generally high sensitivity of *C. tenuicorne* to all tested single substances may be due to the fact that it is a single cell filamentous alga, which means that each cell is in direct contact with the surrounding water and thus exposed to any contaminants.

4.2. Waste waters

The Ceramium growth inhibition test was useful in determining the toxicity of the pulp mill waste waters. A reduction of toxicity before and after secondary treatment of two mills was 60 and 84% respectively. This is consistent with findings by Pokhrel and Viraraghavan (2004) who in a review of aerobic systems for removing harmful soluble organic pollutants from pulp mill effluent water found the efficiencies in reduction between 74 and 90% (Pokhrel and Viraraghavan, 2004). Discharges from pulp mills may contain more than 250 substances (Sreekrishnan and Muna Ali, 2001), which in practice is impossible to determine on each effluent. Biological test methods are thus important complements and may be especially helpful for use on complex waters since they provide single figures for toxic effect of the water.

4.3. Antifouling paints

The *C. tenuicorne* growth inhibition test was able to rank the leachates from antifouling paints according to their toxicity. Five paints were shown not to leach any toxic substances, while some paints claimed as biocide free in fact did leach something that was toxic to organisms naturally living in the coastal environment (Table 2; Karlsson and Eklund., 2004; Karlsson et al.,2006; Ytreberg et al., 2010). These three studies are the only ones where leachates from the whole products of antifouling paints have been tested. The results clearly illustrate the need to make risk assessment not only on the biocides added to a product but to the product as it is used. In particular, products that are designed for leaking chemicals into the environment should be tested and evaluated as a whole product so that any synergistic effects may be detected (Eklund and Karlsson, 2010). In future risk assessments the national competent authorities are urged to require test data from antifouling products as they are used as well as for other products designed for leakage to the surroundings.

4.4. Sediment

Sediments collected from leisure boat harbors clearly showed a negative effect to growth of *C. tenuicorne*. This is in accordance with other studies, e.g. Pane et al. (2008) who studied harbour sediment in Italy and found harmful effects to the alga *Dunaliella tertiolecta* and the invertebrate *Tigriopsus fulvus*. The concentration of contaminated paint flakes particles was quantitatively correlated to the toxic response of the marine macroalga *Ulva lactuca* in a laboratory study, where clean sediment was mixed with contaminated sediment (Turner et al., 2009a). In another study the bioaccessibility and bioavailability of mixtures of sediment and spent antifouling paint particles was tested on the deposit feeder *Arenicola marina* (Turner et al., 2008). In yet another study from the Plymouth group the ingestion of finely ground antifouling paint flakes from a boat yard of the blue mussel *Mytilus edulis* was studied and proved to be negatively affected (Turner et al., 2009b).

The growth inhibition test with *C. tenuicorne* was the most sensitive species of the three species used on sediments from the Stockholm archipelago (the bacterium *Vibrio fisheri* in the Microtox test and larval development of the crustacean *Nitocra spinipes*) and is recommended to be used in future test batteries for testing sediments in the Baltic Sea (Eklund et al., 2010).

A direct comparison between the toxicity results on sediments

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Table 5
Effect values of leachates from boat yard soil to luminiscens inhibition to the bacteria *Vibrio fisheri* (Microtox test), larval development of the crustacean *Nitocra spinipes* and to growth inhibition of the red macroalga *Ceramium tenuicorne*. Samples are from the surface and 20 cm below surface. The tests were performed on pooled samples from three replicates for A, B and C and one sample for D. (Compiled data from tables 7, 8 and 9 from Eklund et al., 2014). The data for Ceramium EC50 values are in this table expressed both as % leachate and as g dw/L. Leachates were produced by sequential dilution of sediment, extraction for 24 h, filtering through 0.45 μm and used in ecotoxicological testing according to preferences of each species.

Soil						
Surface leachate (0–0.5 cm)						
Sample	Microtox	Nitocra		Ceramium		
	Leachate %	Leachate NOEC, %	Leachate LOEC, %	Leachate %	Conf. int. 95%	EC50 g dw/L
70 m from the water, A	165	3	9	1.4	(0.6–2.2)	8.4
90 m from the water, B	NA	NA	NA	1.7	(1.2–2.4)	10
120 m from the water, C	180	45	NA	5.5	(4.4–6.8)	33
160 m from the water, D	16	2	6	0.25	(0–1.0)	1.5
Subsurface leachate (19–21 cm)						
70 m from the water, A	NA	NA	NA	16	(30–48)	92
90 m from the water, B	NA	NA	NA	40	(3.1–7.2)	234
120 m from the water, C	NA	18	18	5.1	(0.27–0.34)	30
160 m from the water, D	41	NA	NA	0.31	(30–48)	1.7

NA – not applicable.

Table 6
Corresponding Cu and Zn concentrations at the EC50 concentration are calculated from analyzed leachate and bold figures denote substances most likely to be responsible for the observed toxicity based on responses to single substances. EC50 for *Ceramium tenuicorne* as single substances in 7 PSU are 3.1 ± 1.1 μg Cu/L and 24.4 ± 5.1 μg Zn/L (Table 1 this article).

Surface leachate (0–0.5 cm)		
Sample	Estimated concentration of Cu and Zn at the EC50 value of the ceramium test	
	Cu μg/l	Zn μg/l
70 m from the water, A	0.83	**28.6**
90 m from the water, B	0.7	**20.8**
120 m from the water, C	**6.5**	**32.2**
160 m from the water, D	2.0	**34.8**
Subsurface leachate (19–21 cm)		
70 m from the water, A	**4.9**	15.0
90 m from the water, B	**13.2**	12.9
120 m from the water, C	2.2	8.8
160 m from the water, D	1.4	15.7

cannot be made as the results have been performed for different reasons and have been expressed in different ways and the content of organic matter may also influence the results. However, high correlation ($R^2 = 0.83$) between toxicity to growth of Ceramium and the content of Cu, Zn and organic tin compounds was found in the harbour sediments (Eklund et al., 2016), which indicates that these compound used in antifouling paints are largely responsible for the effect. Most likely other organisms are also negatively affected. It has been suggested by Molnar (1983), who found high Cu concentrations in the liver of the mute swan, that this was an effect from contaminants from paint flakes.

Not only harbours are highly polluted but also sediments in ship lanes may reach high levels of contaminants affecting organisms (Strand et al., 2003). This was confirmed with the Ceramium study in Stockholm where the toxicity clearly was related to the higher concentrations of organotin compounds, PAHs and metals in the sediment (Table 4B).

4.5. Soil

No other study has been performed on measuring toxicity of soil from boat yards. A study on mussels fed on grounded paint particles from a boat yard (Turner et al., 2009b) is similar but does not consider the paint particles together with the soil. All three test organisms used for testing the soil from a boatyard responded negatively and were able to rank the degree of pollution at the different sites (Eklund et al., 2014). The growth inhibition test with *C. tenuicorne* was the most sensitive test and is a good candidate for future tests of soil (Table 5). In spite that Cu is more toxic to *C. tenuicorne* than Zn when tested as single compounds (Table 1) it was shown that in the soil samples Zn was more often responsible to the toxicity than Cu. This may be explained by the higher water solubility of Zn (Jessop and Turner, 2011), which resulted in one magnitude higher concentrations of Zn in the elutriate water compared to Cu (Eklund et al., 2014). Cu is most likely more hardly bound to the organic matter in the soil. This way of determining the cause of observed toxicities based on results on single substances may be used for other organisms in future studies of waste waters, soil and sediments.

The toxic responses agree well with the high degree of many different contaminants shown by chemical analyses (Eklund et al., 2014). That boat yards often are highly polluted was confirmed by chemical data from 34 investigated boat yards in Sweden where most metals and organic pollutants were above guidance values (Eklund and Eklund, 2014). The ground in all these boat yards consists of gravel and macadam, which is commonly used for winter storage of leisure boats. As the maintenance behavior is similar in many other countries it is likely that these boatyards also are polluted. The risk for leaching to adjacent waters and affecting the coastal eco system is evident. This risk should be considered in management procedure of places for winter storage of leisure boats.

4.6. Comparison of toxicity from antifouling paints, contaminated sediment and boat yard soil

The harmful effects of different boat activities can be evaluated by comparing the % leachate water that is needed to give a 50% growth inhibition to *C. tenuicorne* (Tables 3, 4AB, 5). The antifouling paints produced the most toxic leachate and only 0.17% of leachate from Cruiser superior was sufficient to give a 50% harmful effect. Nine of the other tested paints gave EC50 values with leachate water between 0.19 and 2.8%. This is comparable to the worst sediment found in the center of the city with an EC50 of 0.9% and closest to the slipway with an EC50 of 1.3%. Also the sediments in the ship lane at 1, 15 and 25 km from the center were highly toxic and 1.4–3% leachate water was sufficient to reduce growth of

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Ceramium by half. The soil also produced leachates in the same toxicity range and six out of the eight tests, produced EC50 values of 0.31–5.5% leachate waters. The conclusion is that all boat activities are in the same range when it comes to toxicity and it is recommended to introduce regulations to minimize further pollution. The occurrence of chemicals derived from the use of antifouling paints has been measured in the environment at numerous places in detectable levels around the world in water and sediment (e.g. Hoch, 2001; Thomas et al., 2001; Kobayashi and Okamura, 2002; Konstantinou and Albanis, 2004; Fent, 2006; Oliviera et al., 2010; Turner, 2010). A thorough review on environmental levels, toxicity and human exposure to tributyltin (TBT) was published by Antizar-Ladislao (2008) which contains environmental levels, toxicity and human exposure to TBT in the marine environment. In a recent review of ecological strategies and impacts of antifouling paints, directions for the management of antifouling paints was highlighted (Dafforn et al., 2011). Improvements of management of boat yards have been suggested by Eklund and Eklund (2014). All studies point at the hazard of antifouling paints and the importance of minimizing discharge and spread of toxic substances in the environment in connection with boat activities.

5. Conclusion

The growth inhibition test with the macroalga *C. tenuicorne* is suitable for use in different types of matrices such as water, sediment and soil and different types of applications. The standardization of the growth inhibition method with *C. tenuicorne*, the wide tolerance of salinities (4–32‰) and thus relevance for large areas, makes the test useful within several regulations, e.g. REACH, BPR, Marine Strategy Framework Directive (MSFD) and Water Framwork Directive (WFD). The high sensitivity, high reproducability, low cost and the robustnes of the species are a further advantages of the test method. The test was able to rank the different tests/samples according to their toxicity. A test on macroalgae is particularly useful since an adverse impact on macroalgae means an effect early in the food web, which implies secondary effects on higher trophic levels.

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Please cite this article in press as: Eklund, B., Review of the use of *Ceramium tenuicorne* growth inhibition test for testing toxicity of substances, effluents, products sediment and soil, Estuarine, Coastal and Shelf Science (2016), http://dx.doi.org/10.1016/j.ecss.2016.10.009